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by PML in Human Breast Cancer

PRINCIPAL INVESTIGATOR: Khew-Voon Chin, Ph.D.

CONTRACTING ORGANIZATION: Cancer Institute of New Jersey  
Piscataway, New Jersey 08854-5638

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## Introduction

Breast cancer is one of the most prevalent malignancies in women and accounts for the highest morbidity among women suffering from cancers (1). The oncogenic development of breast cancer is accompanied by genetic alterations of multiple oncogenes, tumor suppressor genes, and other factors. The collaborative effects of these transforming proteins induce alterations in the cellular biochemical, physiological, and genetic processes, which include both gene induction and gene repression, alterations in growth requirement, and acquisition of metastatic potential. These changes may lead to neoplastic transformation of the mammary tissue. The complexity and heterogeneity of the array of genetic, hormonal, and dietary factors that may contribute to the etiology of breast cancer is further confounded by the lack of information on specific genetic mutations associated with the initiation and progression of the disease.

Overexpression of the epidermal growth factor receptor (EGFR), *HER-2/neu*, and *myc* oncogenes are some of the well described genetic changes that frequently occur in breast cancer (2-4). In addition, deletions of chromosomal loci that are thought to be associated with putative tumor suppressors including the *p53*, *BRCA1* and *BRCA2* genes, also contribute to a more aggressive phenotype of breast cancer (5). These genetic changes have important prognostic implication in the clinical outcome of breast cancer.

Epidermal growth factor (EGF) is a small polypeptide that stimulates cell proliferation in both cell culture and in intact animals (6). EGF has been shown to promote both normal and neoplastic growth of mammary tissue in rodents (7) and in human breast cancer cells in culture (8-10). The biological effects of EGF are mediated through high affinity binding to EGFR, which is a 170 kDa membrane receptor tyrosine kinase (6). There is great interest in the study of EGFR in human breast cancer, however, the clinical relationships and prognostic value of the receptor in breast cancer are still unclear (11).

The transforming growth factor- $\alpha$  (TGF $\alpha$ ), which bears considerable sequence homology to EGF and is produced by many transformed cells, also binds to EGFR and mimics the action of EGF (6,12). Expression of EGFR in breast cancer cells is regulated by mitogenic growth factors, and also by the superfamily of nuclear hormone receptors, which includes estrogen, progesterone, glucocorticoids, and retinoic acid receptors (13). Therefore, optimal regulation of EGFR expression is a complex process involving the coordinate interaction of several heterologous growth factors and hormones, whereby the proliferation of normal and neoplastic breast cells can be modulated. Although the clinical significance and prognostic value of EGFR in human breast cancer are unresolved, the involvement of EGFR in the growth of normal and malignant human mammary cells indicates that it may play a critical role in the oncogenesis of human breast carcinomas. Further evidence of the importance of EGFR in the development of breast cancer has been shown in transgenic mice studies where the overexpression of TGF $\alpha$  causes a significant increase in the occurrence of mammary carcinomas (14-16).

Specific chromosomal abnormalities occur frequently in acute and chronic leukemias (17). These cytogenetic aberrations are thought to contribute to leukemogenesis. Furthermore, significant differences in the type of genes involved in chromosomal translocations in acute leukemias and chronic leukemias have also been observed. For example, study of the recombination of *bcr* and *abl* genes in the t(9;22) of CML, and the *myc* and immunoglobulin genes in the t(8;14) of Burkitt lymphoma, has led to the identification of new fusion genes involved in the neoplastic transformation of these hematopoietic tumors (17-19). It has also been shown that a chromosomal translocation breakpoint t(15;17) occurs in over 90% of all patients with acute promyelocytic leukemia (APL), a subtype of acute myeloblastic leukemia (20-23). The recombination involves the *PML* (*myl*) gene on chromosome 15 and the retinoic acid receptor- $\alpha$  (RAR $\alpha$ ) on chromosome 17. The chimera *PML/RAR $\alpha$*  and *RAR $\alpha$ /PML* genes are formed as a result of the reciprocal translocation between the *PML* and RAR $\alpha$  loci (20-22, 24). The *PML/RAR $\alpha$*  cDNA has been isolated and shown to encode a fusion protein that is retinoic acid

responsive and exhibits transactivation potential in a cell type- and promoter-specific manner differing from the wild-type RAR $\alpha$  (25-27). Since the administration of all-trans retinoic acid to APL patients leads to rapid achievement of remission, it has been suggested that disruption of RAR $\alpha$  may be part of the underlying pathogenesis of APL (28-30). The biological function and etiologic implications for the *PML* gene is not known and leaves open the question of its role in APL. Characterization of *PML* reveals that it is a putative zinc finger protein and transcription factor that shares homology with a newly recognized family of proteins that includes a variety of putative transcription factors as well as the recombination-activating gene product (RAG-1) (25-27,31). Expression of *PML* is found in a variety of fetal and adult tissues including brain, gut, liver, lung, muscle, placenta, and testes (31,32).

Our laboratory, in collaboration with Dr. Kun-Sang Chang at the University of Texas, M.D. Anderson Cancer Center, investigated the role of t(15;17) chromosomal translocation in the leukemogenesis and the emergence of multidrug resistance in acute promyelocytic leukemia (APL). We demonstrated that *PML* suppresses the clonogenicity and tumorigenicity of the APL-derived NB4 cells in soft agar (33). Cells transfected with expression vector containing *PML* showed more than 50% reduction in colonies formed on soft agar. Cells transfected with control plasmid (pSG5) and *PML* mutants (PSG5*PML*mut and pSG5*PML*/RAR $\alpha$ ) did not show inhibition of colony growth. Furthermore, we also show that *PML* suppresses the transformation of REF and NIH3T3 cells by oncogenes. In all of these experiments the fusion product *PML*/RAR $\alpha$  fails to suppress the tumorigenic growth of NB4 cells as well as the transformation of the REF and NIH3T3 cells. These results suggest that the translocation of APL inactivated the biological function of *PML* as a tumor suppressor and that this molecular alteration may be a precipitating event in the development of APL.

To assess the putative function of *PML* and *PML*/RAR $\alpha$  as a transcription factor, we examined their ability to transactivate promoter activity. In this study, we demonstrated that *PML*/RAR $\alpha$  can transactivate from the p21<sup>WAF1/CIP1</sup> promoter in an RA-dependent manner. Deletion analysis revealed a region in the promoter between -94 and -66 relative from the transcription start site that is required for transactivation by *PML*/RAR $\alpha$ . When this region was fused to a heterologous HSV TK minimal promoter, it can confer *PML*/RAR $\alpha$  stimulation, with further response to RA.

### Body

In this grant period (August 1, 1996 - July 31, 1997), to determine whether *PML*/RAR $\alpha$  may stimulate the transcription of the p21<sup>WAF1/CIP1</sup> gene, a construct containing 2.4 kb of the p21<sup>WAF1/CIP1</sup> promoter placed in front of a bacterial chloramphenicol acetyltransferase (CAT) reporter gene, was transiently cotransfected with the *PML*/RAR $\alpha$  expression plasmid into a human breast carcinoma SK-Br-3 cells. The SK-Br-3 cells are p53<sup>-/-</sup>, thus eliminating any potential transactivation of the p21<sup>WAF1/CIP1</sup> promoter reporter by p53. Figure 1 (representative results from one of five transfection experiments) shows that the expression of *PML*/RAR $\alpha$  in SK-Br-3 cells resulted in an induction of the CAT activity in comparison with the pSG5 vector control. The induction was further increased to approximately 13-fold when the transfected cells were treated with RA for 24 h. The induction of the CAT activity by RA was mediated by *PML*/RAR $\alpha$ , since addition of RA did not result in increased CAT activity. Consistent with other reports, our results also indicate that *PML*/RAR $\alpha$  may be a ligand-binding transcription factor (26,27). These results indicate that the p21<sup>WAF1/CIP1</sup> gene may be a target for *PML*/RAR $\alpha$  and the activation is RA responsive.

We next examined whether it was the *PML* or the RAR $\alpha$  moiety of the fusion protein that activated the p21<sup>WAF1/CIP1</sup> promoter activity. Moderate transactivation of the promoter was observed with *PML* in SK-Br-3 transfected cells but no further alterations in the CAT activity was

observed in the presence of RA (Fig. 1). However, no significant activation of the p21<sup>WAF1/CIP1</sup> promoter was observed with RAR $\alpha$ , either in the presence or absence of RA (Fig. 1). Taken together, these results suggest that the activation of the p21<sup>WAF1/CIP1</sup> promoter by PML/RAR $\alpha$  is not attributable to the domain from RAR $\alpha$ . This gain of function may be the result of a conformational change and activation of the transcriptional activity of the PML moiety of the PML/RAR $\alpha$  fusion protein, thus enabling it to alter p21<sup>WAF1/CIP1</sup> gene activities. As controls, we also examined the effects of p53 on p21<sup>WAF1/CIP1</sup> promoter in H1299 cells. Clearly, expression of wild type p53 in H1299 resulted in a strong induction of the p21<sup>WAF1/CIP1</sup> promoter activity while a mutant p53 failed to transactivate it (Fig. 1).

### Localization of PML/RAR $\alpha$ response element in the p21 promoter

Since PML/RAR $\alpha$  can transactivate the p21<sup>WAF1/CIP1</sup> promoter, we performed deletion analysis to determine the putative PML/RAR $\alpha$  response element in the p21<sup>WAF1/CIP1</sup> promoter using a series of deletion mutants cloned into a luciferase reporter vector<sup>12</sup> (Fig. 2). The deletion constructs were cotransfected with the PML/RAR $\alpha$  expression plasmid into cells either in the presence or absence of RA. As shown in Fig. 2, progressive deletion of the p21 promoter up to nucleotide -121, relative from the transcription start site, conferred response to activation by PML/RAR $\alpha$  and in the presence of RA. However, deletion of the promoter sequences downstream of -94 abolished the promoter activity and transactivation by PML/RAR $\alpha$  (Fig. 2). These results suggest that the sequence between -94 and -61 is required for transactivation by PML/RAR $\alpha$  in the presence of RA (Fig. 2). Since this region is also essential for basal promoter function, therefore, PML/RAR $\alpha$  may interact with the basal transcription factors to activate p21<sup>WAF1/CIP1</sup> gene transcription.

To further verify this PML/RAR $\alpha$  response element, we placed the promoter sequence between -94 and -66 immediately upstream of the minimal HSV thymidine kinase promoter, which drives the expression of a CAT reporter gene (Fig. 3A). The PML/RAR $\alpha$  response element conferred approximately 4-fold increase in promoter activity and further induction was observed in the presence of RA (Fig. 3B). The vector control without PML/RAR $\alpha$  had no effect on the promoter activity (Fig. 3B). These results suggest that the region between -94 and -66 can confer stimulation by PML/RAR $\alpha$ , and the response is further increased in the presence of RA.

### Conclusions

The universal cell cycle inhibitor p21<sup>WAF1/CIP1</sup> was first identified as a target gene for the tumor suppressor p53<sup>16</sup>. In this study, we demonstrated that PML/RAR $\alpha$  can transactivate from the p21<sup>WAF1/CIP1</sup> promoter in an RA-dependent manner. Deletion analysis revealed a region in the promoter between -94 and -66 relative from the transcription start site that is required for transactivation by PML/RAR $\alpha$ . When this region was fused to a heterologous HSV TK minimal promoter, it can confer PML/RAR $\alpha$  stimulation, with further response to RA. Therefore, genetic alterations of *PML* including gene mutations, and aberrant chromosomal translocation such as those in acute promyelogenous leukemia involving RAR $\alpha$ , may be common in breast cancer. Indeed expression of *PML* has been shown to be altered in breast cancer (34). In normal breast specimens, less than 3% of the epithelial cells exhibit *PML* staining, but increasing levels of *PML* was detected as the lesions progress from benign dysplasias to carcinomas. *PML* expression is also markedly reduced when malignant cells become invasive (34). These studies further suggest that *PML* may play a critical role in breast carcinogenesis. We reasoned that aberrant regulation of p21 by *PML* may contribute to the development of breast cancer. Future studies will involve the characterization of the regulatory relationship between *PML* and p21.

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## Appendix

**Fig. 1.** Transcription activation of p21 by PML/RAR $\alpha$ . SK-Br-3 cells were cotransfected with p21-CAT and the indicated expression plasmids in the presence or absence of 1  $\mu$ M RA. The amount of DNA in each cotransfection was kept constant through the addition of ssDNA. RA was added 16 h after transfection. The cells were harvested 40 h after transfection and assayed for CAT enzyme activities, which were normalized for cellular protein concentration. Shown is a representative experiment done at least five times.

**Fig. 2.** Deletion analysis of the p21 promoter. Full-length and deletion mutant of p21 promoter reporter constructs were cotransfected with PML/RAR $\alpha$  expression plasmid in the presence or the absence of 1  $\mu$ M RA. "TATA" represents the p21 TATA box located 45 bp from the transcription start site (defined as +1). The 5'-boundaries (bp upstream of p21 transcription initiation site) of the reporters are indicated to the left of each construct, and all the constructs shown shared the same 3'-boundary located at +16 downstream of the transcription start site.

**Fig. 3.** Stimulation of transcription by PML/RAR $\alpha$  response element within the p21 promoter. (A) The p21 promoter sequence between -94 and -66 fused to pBLCAT5. TKp, thymidine kinase promoter from herpes simplex virus gene; CAT, gene encoding CAT. (B) Plasmids pBLCAT5 or the construct in (A) were cotransfected with the PML/RAR $\alpha$  expression plasmid in the presence or absence of 1  $\mu$ M RA. The CAT activity was measured and fold induction was determined by comparing normalized CAT activity in cells transfected with the PML/RAR $\alpha$  expression plasmid to cells transfected with the control vector pSG5.

Figure 1

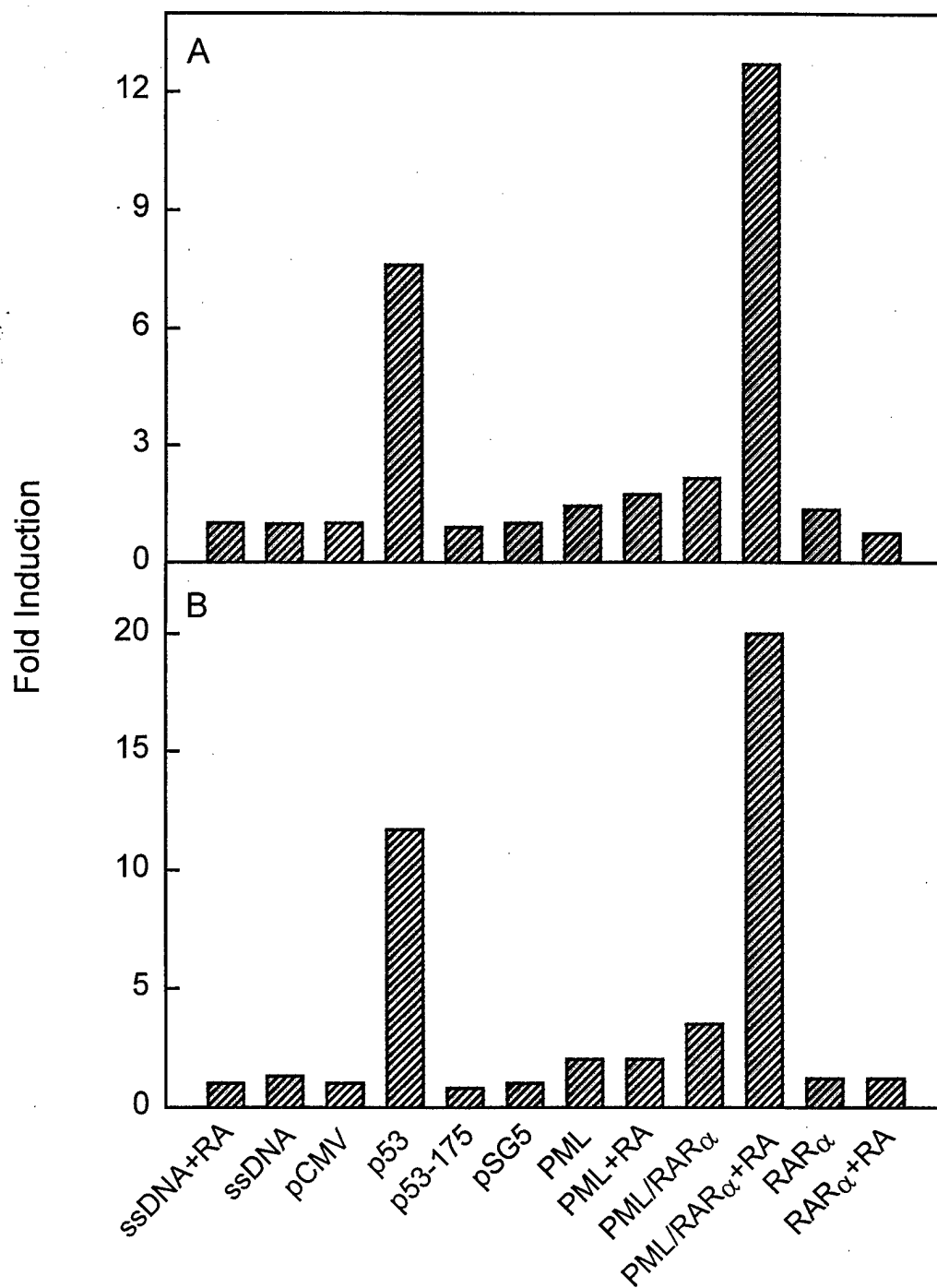
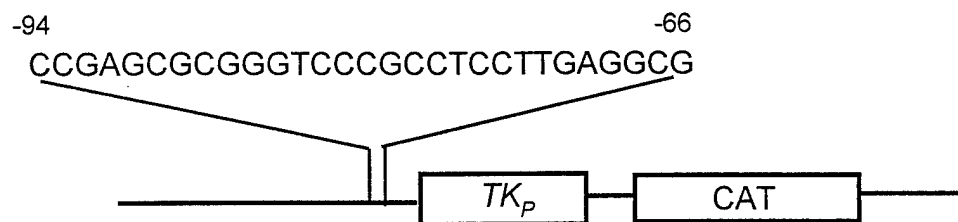


Figure 2

A



B

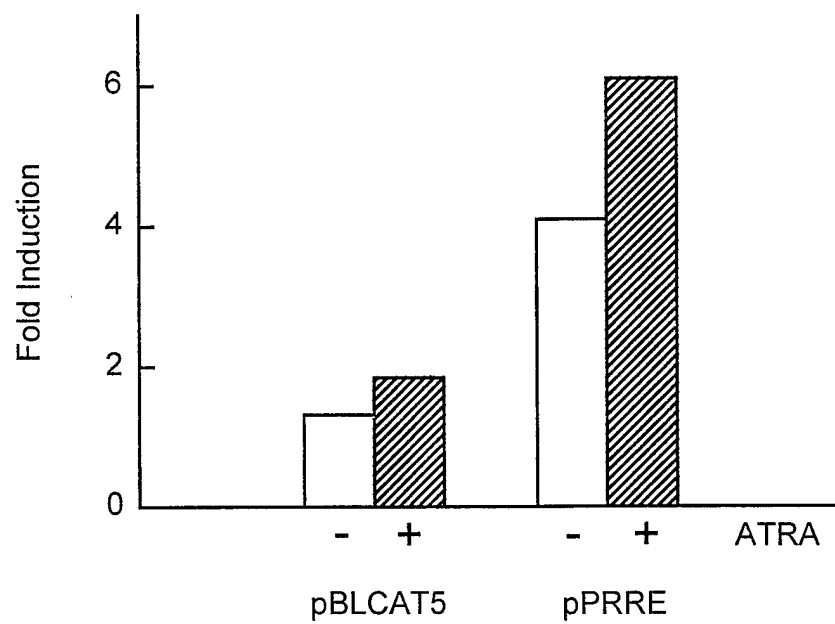
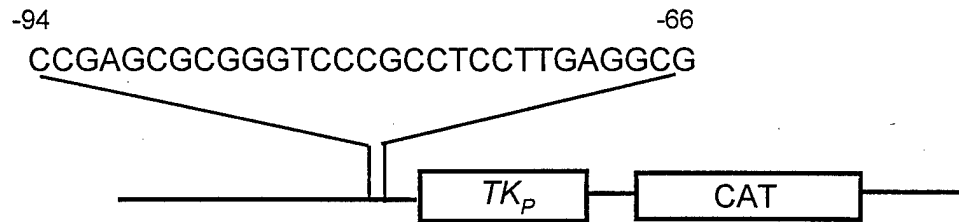


Figure 3

A



B

